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General Introduction

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Introduction

Sexual reproduction presents organisms with the unavoidable challenge to select a conspecific in order to reproduce. For most species, this necessitates interaction between a male and a female. As the sexes are fundamentally distinct forms, their role in the process of mate selection and progeny production is, of course, also dissimilar. It is well established that pre-copulatory mate selection, in most species, typically involves males indicating interest by displaying stereotypic species-specific courtship behaviour, and females via accepting or rejecting (Parker and Birkhead, 2013). Broadly speaking and grossly over simplifying, males are regarded as the gaudy, visually enticing sex; and females the choosey. During mating the differences between the sexes are complementary as males and females need each other in order to be represented genetically in future generations, and mating benefits both males and females by increasing their reproductive output. However, after mating the different interests of males and females become a source of conflict: a sort of trouble in paradise.

Sexual conflict theory (Chapman et al., 2003a) suggests that sex-differences give rise to male- and female-specific costs and benefits associated with reproduction. And therefore, males and females achieve optimal personal reproductive success in different ways. Conflict arises when one of the sexes pursues personal reproductive success at a cost to their previous mate. A classic example of this is sexual promiscuity. Achieving multiple mates is largely beneficial for the individual's reproductive success. When a male mates with multiple females, it directly increases his probability of producing offspring (Janicke et al., 2016). Although this relationship does not directly transpose to females, they still benefit from extra sexual activity (reviewed by Gowaty, 2012; Jennions and Petrie, 2000; Parker and Birkhead, 2013; Slatyer et al., 2011). Females may mate with multiple males because it costs less than constantly displaying rejection behaviour (convenience polyandry; Rowe, 1992), they directly benefit from polyandry by gaining resources (such as nuptial gifts), protection and certainty of fertilization (briefly reviewed in Jennions and Petrie, 2000), or they increase the quality or quantity of the offspring produced (Arnqvist and Nilsson, 2000; Gowaty, 2012). As females of most species naturally mate with several males over their lifespan, the benefits of polyandry are regarded as nearly universal (for review see Gowaty, 2012).

Although mating can potentially be costly to the individual through increased exposure to sexually transmitted diseases (Otti, 2015), increased predation risk (Hartbauer and Römer, 2016), and even lowered lifespan (Gems and Riddle, 1996) (but see Gowaty et al., 2010; Markow, 2011), these costs are outweighed by the

benefits of reproduction. However, as sex is a fundamentally social interaction, an individual's behaviour can also reduce the reproductive success of their previous sexual partner(s), and this is particularly true for female remating behaviour. While polygyny rarely comes at a cost to the male's earlier female conquests (except in the case or reduced paternal care), polyandry on the other hand incurs large costs to her previous mates as it drastically reduces the number of offspring they sire, creating conflict over female post-mating behaviours.

SEXUAL CONFLICT OVER POLYANDRY

The cost that males pay to polyandry has a lot to do with asymmetry in gamete production. Relative to sperm, eggs are expensive to produce; this means that males can make a much larger supply of gametes compared to females. Consequently, when a female mates, once or multiply, she acquires much more sperm than she has eggs to fertilize. Therefore, the relationship between female mating rate and offspring production is neither linear nor additive: remating does not usually result in a doubling of offspring production. If it did, polyandry would not reduce the female's previous mate's chance of paternity. Rather, female remating causes a redistribution of paternity through the process of post-copulatory sexual selection. With respect to each male, this reduces the amount of offspring sired and therefore a cost to each males' reproductive success, creating conflict between the sexes for optimal female mating rate.

Sexual conflict theory (Chapman et al., 2003a) predicts that males should develop, over evolutionary time, adaptations to reduce polyandry or reduce the costs they pay due to it. Any new adaptation that males evolve becomes a part of the social environment in which females are also evolving; and, in turn, females should develop counter-adaptations to again increase polyandry. Consequently, a new rise in polyandry would again instigate male-produced adaptations to reduce such behaviour. In other words, the appearance of traits that influence polyandry will apply new selective pressures resulting in the development, over evolutionary time, of another counter-adaptation in the other sex (Moore and Pizzari, 2005). This results in a permanent cycle of adaptation/counter-adaptation, which is predicted to speed up the evolutionary process (Moore and Pizzari, 2005). An example of this cycle is presented in Figure 1 (Moore and Pizzari, 2005).

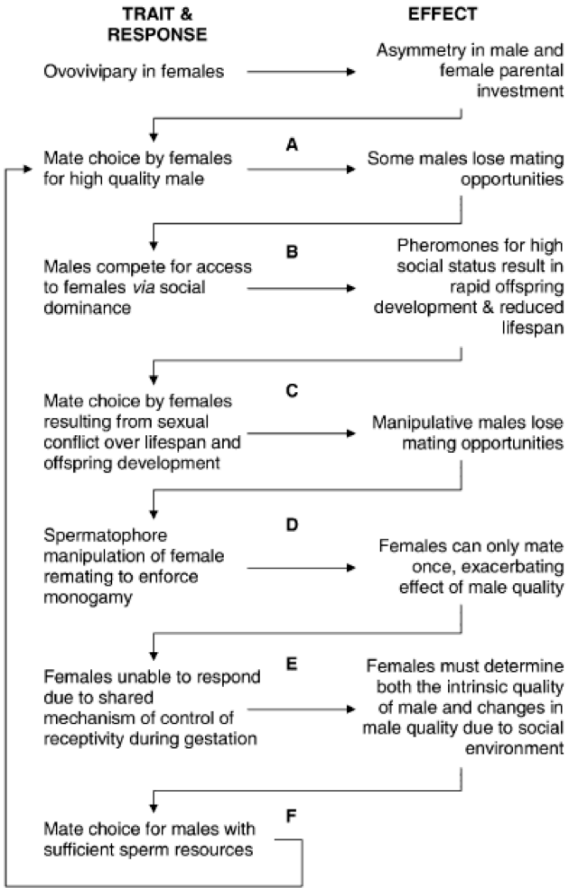

Figure 1. Flow chart of adaptation counter-adaptation. Example of how sexual conflict and sexual selection may result in the evolution of a series of adaptations and counteradaptations in males and females of the cockroach *Nauphoeta cinerea*, a species that is nearly a model system for studies of sexual selection and sexual conflict. This flow diagram represents a hypothesis for the evolution of the multiple traits previously shown in separate studies to have evolved in response to sexual selection and sexual conflict. Taken from Moore and Pizzari, 2005.

INVESTIGATING SEXUAL ANTAGONISTIC CO-EVOLUTION IN *DROSOPHILA*

Female polyandry and the male-derived traits that evolve as a response to reduce polyandry are both sexually antagonistic traits, meaning that they benefit the individual at a cost to their social partner(s). Some of the largest advances in understanding the mechanisms that underlie such traits came from research using the model organism *Drosophila melanogaster*. Females of this species are naturally polyandrous as shown by the observation that females collected in the wild always have sperm from multiple males within their sperm storage organs (Harshman and Clark, 1998; Imhof et al., 1998; Markow, 2011, for a review see Gowaty, 2012). Moreover, females in the lab accept multiple partners per day under conditions

where food is available (Billeter et al., 2012; Gorter et al., 2016; Markow, 2002; Prout and Bundgaard, 1977). Critically, when females re-mate, each partner rarely achieves equal proportion of sires; it is usually the sperm from the second male that is used to produce the majority of the offspring (about 80-90%; Clark et al., 1995). This phenomenon, known as last male sperm precedence (LMSP; Clark et al., 1995), demonstrates a particularly high cost of polyandry to males since they may lose up to 90% of their fecundity.

The main advantage of utilizing this species in research is making use of the wide array of genetic tools readily available. This allows testing specific hypotheses generated by sexual conflict theory to determine not only *if* males attempt to influence polyandry and/or secure their chances of paternity in a multiply mated female, but also *how* they go about doing this. Thus, research on *D. melanogaster* has the potential to fulfill the holy grail of biology: linking ultimate explanations about the function of a behaviour with the proximate mechanisms explaining how that particular behaviour is generated.

As polyandry is costly to males, sexual conflict theory predicts that *Drosophila* males should either develop mechanisms that reduce their partners' ability to re-mate and/or increase the chances of the female using his sperm over that of another male. Research that has focused on male adaptations to reduce the cost of polyandry has yielded unparalleled access into mechanisms supporting reproduction (addressed below, in "Male tactics and female response"). However, sexual conflict theory also predicts an adaptation/counter-adaptation evolutionary arms race between the sexes (Moore and Pizzari, 2005). Unfortunately, the amount of literature investigating the female response pales in comparison to male-centered examinations, effectively limiting our understanding of the interactive phenomenon. This male bias in research is not exclusive to polyandry but occurs in other reproductive traits where there is a clear complex co-evolutionary dynamic relationship (Ah-King et al., 2014). The focus on male-influencing variables in general has inadvertently formed a bias in the research implying that females fall victim to male strategies or are mere arenas for contests, such as sperm competition, to take place. As the development of female responses to male-derived adaptations are theoretically anticipated (Alonzo and Pizzari, 2013; Moore and Pizzari, 2005), a full understanding of both male tactics and female counter-strategies is required. We have limited our knowledge in this area when we have neglected to explore the female within this interaction. As it is indeed more difficult to understand the female contribution, a good strategy is then to piggy-back on the foundation of knowledge of the male-derived tactics, within the *Drosophila* model organism, and see how females contribute to this process.

In this thesis, I have directly explored the female's response to male tactics to decrease polyandry and monopolize the paternity of offspring with the goal to advance our understanding of how female post-copulatory behaviour contributes to her reproductive success. Basically, I ask: do females have any say in the matter, and if yes, how do they express themselves?

FEMALE POST-MATING RESPONSE

In order to determine if females have indeed developed counter-adaptations to male tactics aimed at monopolizing female reproduction, it is important to not only identify typical female post-mating behaviours but also to understand how these behaviours are generated. In **Chapter 2** of this thesis, I review in depth the various behaviours that make up the female post-mating response (PMR) along with the neuronal circuitry that supports these behaviours. Mated *D. melanogaster* females have a well-characterized PMR including decreased sexual receptivity and increased egg-related behaviours (ovulation and ovipositioning Manning, 1967). The initiator of some of this transformation is a small peptide, known as sex peptide (SP), transferred to females along with sperm within the ejaculate during copulation (Chen et al., 1988; Peng et al., 2005a). Although investigations involving the PMR have focused on sexual receptivity and egg laying behaviour, I highlight several other behaviours that have also been correlated with this change in mating status. For instance, mated females display very different sleep/wake patterns (Isaac et al., 2010), and food preferences (Ribeiro and Dickson, 2010) compared to virgin females. Furthermore, in addition to these more overt behaviours, *Drosophila* females also show great control over the movement and utilization of the ejaculate within their reproductive tract (Adams and Wolfner, 2007) including taking what they need (sperm storage; Arthur et al., 1998) and removing what they don't (sperm ejection; Lee et al., 2015; Manier et al., 2010; Snook and Hosken, 2004). Identification of the individual behaviours that together form the PMR as well as research into the cellular mechanisms that support them has revealed a large overlap in first order neurons involved in the circuitry that support its various aspects (Rezával et al., 2012; Hasemeyer et al., 2009; Yang et al., 2009; Yapici et al., 2008). This indicates that females experience a general transition of behavioural states rather than the initiation of individual aspects of the PMR in isolation.

Of the various post-mating behaviours exhibited by females, some represent behaviours that benefit both sexes by increasing general reproductive output while others exemplify those that counteract male-ploys to restrict remating. In this thesis I explore the female responses to two male tactics by identifying which females

post-mating behaviours may counteract male exploits and the neuronal circuitry supporting this female control.

MALE TACTICS AND FEMALE RESPONSES

Drosophila males manipulate female behaviour in ways that increase their own reproductive success. To reduce the chances of future infidelities by their previous mates, males transfer chemicals to females during mating: some reducing their sexual receptivity (Avila et al., 2011); and other reducing their attractiveness and therefore courtship by potential mates (Jallon, 1984; Zawistowski and Richmond, 1986), a phenomenon known as chemical mate-guarding. In the event of remating, males also reduce the cost of polyandry by also transferring substances within the ejaculate that function to increase male reproductive success (Bretman et al., 2009; Wigby et al., 2009). In this thesis, I examine female behavioural response to chemical mate-guarding; as well as overt and cryptic responses to sperm competition.

Chemical mate-guarding and sperm ejection

During copulation, males transfer chemicals to females, which alter their pheromonal profile. These changes function to reduce attractiveness, inhibiting courtship and deterring male interest- therefore decreasing chances of female remating. Chemical mate-guarding has been extensively studied in *Drosophila* leading to the identification of the male derived pheromones involved in this phenomenon. The main pheromone that inhibits courtship is cis-vaccenyl acetate (cVA; Jallon, 1984; Mane et al., 1983; Zawistowski and Richmond, 1986). Moreover, researchers have also identified the male neuronal circuitry supporting courtship inhibition in response to cVA pheromones (Datta et al., 2008; Ejima et al., 2007; Kohl et al., 2013; Kurtovic et al., 2007; Ruta et al., 2010). As polyandry is beneficial for females, and a reduction in chances of remating or limiting potential partners would therefore be costly, sexual conflict theory predicts that females should develop a mechanism to remove or deactivate the pheromones used in chemical mate-guarding to restore her attractiveness and increase the chances of remating. However, no such mechanism for female counter-adaptation had been reported.

cVA is produced in the male reproductive tract and transferred to females via the ejaculate (Butterworth, 1969). Interestingly, a few hours after mating females

remove unused portions of the ejaculate including the mating plug and unstored sperm via sperm ejection (Lee et al., 2015; Lüpold et al., 2013; Manier et al., 2010; Snook and Hosken, 2004). Moreover, previous literature documents a significant decrease in this compound around the same time but suggested that the time-dependent reduction may be due to enzyme degradation (Mane et al., 1983). Instead, in **Chapter 3**, I show that the ejected sperm mass indeed contains not only sperm but also high levels of cVA. The consequential removal of this compound and chemicals indicates that sperm ejection can also function to remove anti-aphrodisiac pheromones, which influence post-mating attractiveness. The relationship between increasing attractiveness and polyandry is crucial to establishing sperm ejection as a mechanism of female control over reproduction. This control could have serious fitness consequences as the timing of remating and number of partners could have a large impact on progeny.

There is some evidence that sperm ejection is indeed an active process as neurons involved in the signaling pathways of diuretic hormone 44 have been shown to influence sperm ejection behaviour (Lee et al., 2015) and the timing of sperm ejection is influenced by the social context of the female (Laturney and Billeter, 2016, **Chapter 3**). In **Chapter 6**, I explore the involvement of the CNS in sperm ejection and identify neuronal populations that may be involved in this process.

Sperm competition and remating

When females re-mate, the ejaculates from multiple males interact within the female reproductive tract and compete for a place in one of the two sperm storage organs (SSOs), and ultimately for a chance at fertilization. The typical outcome of this interaction is LMSP (mentioned above) as most studies report ~80% of offspring from the last male. Most of what is known about LMSP in this species comes from laboratory studies that make use of a very specific paradigm. Researchers first mate a female, isolate her for 1-5 days, then mate her with a phenotypically distinct male. The progeny after the second mating is collected and sorted based on phenotype. These patterns of paternity have been explored including a comprehensive investigation into factors that influence LMSP such as male-derived peptides that influence a male's success in this interaction (Avila et al., 2011).

Given that polyandry is common, there should be strong selection pressure on males to develop either offensive or defensive abilities during sperm competition. Indeed, natural genetic variation in seminal fluid protein genes (Clark et al., 1995),

genetic mutants (Gilbert and Richmond, 1981), sperm traits (Lüpold et al., 2012; Manier et al., 2010), exposure to chemicals (Misra et al., 2014), and different social contexts (Bretman et al., 2013; Moatt et al., 2014) have all been found to influence the chances of male success in the battle of ejaculates. Plasticity has also been found with males being able to manipulate ejaculate composition based on perceived sperm competition (Wigby et al., 2009; Garbaczewska et al., 2013) and female mating history (Sirot et al., 2011) suggesting that seminal fluid components do indeed function in sperm competition to increases chances of paternity.

Due to LMSP, female remating appears to mostly change the paternity of the offspring instead of making them more genetically varied as most offspring will descend from the same mate. As increasing genetic diversity of offspring is one proposed benefit of polyandry, females should develop mechanisms to modulate outcomes of sperm competition in order to maximize reproductive success. There are numerous studies that suggest females do influence sperm competition. Female genotype (Chow et al., 2010; 2012; Clark and Begun, 1998; Clark et al., 1999; Giardina et al., 2011), age (Mack et al., 2003), morphology (Amitin and Pitnick, 2007; Miller and Pitnick, 2003), and even social context (Billeter et al., 2012) all influence the paternity of offspring, but no mechanisms has been identified. In other species, the amount of time between copulation and the number of male partners influence LMSP, indicating that mating rate may contribute to a female's ability to modulate paternity patterns. However, the current paradigm in use in *Drosophila* holds female mating rate steady. Recently, one study altered the classic paradigm to include a group of flies (6 males, 6 females) held continuously together for 8 hours, allowing for variation in mating rate. Interestingly, they also reported a breakdown in LMSP after 3 matings (Billeter et al., 2012). If females can modulate the genetic diversity of their offspring by modifying remating behaviour, then this classic paradigm would fail to capture this. In **Chapter 4**, I explore the effect of these two variables (number of mates and length of remating latency) on LMSP by modifying the paradigm in which it is tested and show that female mating rate can indeed lead to a breakdown in last male sperm precedence.

Sperm competition and sperm storage

Besides overt post-copulatory mating behaviour, females may also have hidden control over the fate of sperm in the reproductive tract. Females that could bias sperm storage or patterns of paternity would be at a large selective advantage as they would be able to maximize reproductive success even in the face of sperm

competition by ensuring genetic diversity, genetic quality, or simply quantity of offspring produced (Jennions and Petrie, 2000). Post-copulatory mate choice, or cryptic female choice (CFC), is paternity selection that occurs within the reproductive tract of females who have multiply-mated. Either through biochemically or mechanically mediated processes, the female selects the sperm that will or will not fertilize her eggs.

CFC may be basic in theory (controlling the fate of sperm) but it is diverse in practice. Evidence that females can affect the paternity of her offspring is found in a variety of species including both vertebrates (feral fowl, Pizzari and Birkhead, 2000), and invertebrates (Brazilian beetles, Wilson et al., 1997); and each species could potentially manipulate the sperm in a completely different fashion. In general, for a female mediated event to be viewed as cryptic choice it must account for a unique portion of variance that is not explained by male mediated events such as sperm competition (Eberhard, 1996) and also have an active female component (Telford and Jennions, 1998) where females discriminate on specific male trait(s), and bias paternity accordingly (Eberhard, 1996). In *Drosophila*, although females contribute to variation in sperm competition outcomes (see above), the identification of the full active female component is still missing. Thus, establishing such a mechanism in *Drosophila* requires not only determining how females select paternity within the reproductive tract, but also the sensory system sensitive to the variation in male traits and the neuronal circuitry connecting the two.

Although this is a tall order, even for the well-equipped toolbox of a Drosophilist, evidence is beginning to mount suggesting that the female central nervous system is involved with paternity biasing. A genome wide association study identified female genetic variation that correlated to phenotypic variation in the outcome of sperm competition. Interestingly, half of the genes identified are highly expressed in the nervous system and have neuron-specific functions (Chow et al., 2012). When a subset of these genes were knocked-down in a specific neuronal population innervating the female reproductive tract it caused females to produce different patterns of paternity compared to controls (Chow et al., 2012), suggesting that neurons influence the fate of sperm within her reproductive tract. These neurons may function to control the outcome of sperm competition by influencing sperm storage/displacement or sperm ejection (Lüpold et al., 2012; Manier et al., 2010). Therefore, the identification of the neuronal circuitry supporting these two sperm manipulating processes would provide the first step in identifying the active component responsible for female-mediated sperm precedence and evidence for CFC in this species.

Females store sperm within sperm storage organs: one long coiled tube called the seminal receptacle and two mushroom-shaped spermathecae (Figure 2). Although this process requires an intact female central nervous system (Arthur et al., 1998) as well as specific neuromodulators (octopamine and tyramine) to trigger sperm usage (Avila et al., 2011), the neuronal circuitry supporting sperm storage remains elusive.

Taken together with previous sperm competition experiments, it is clear that females have the potential to control the process of sperm storage and release; and ultimately, may have control over the paternity of her offspring. In **Chapter 5** and **Chapter 6**, I explore the involvement of the CNS in sperm storage and sperm ejection, respectively.

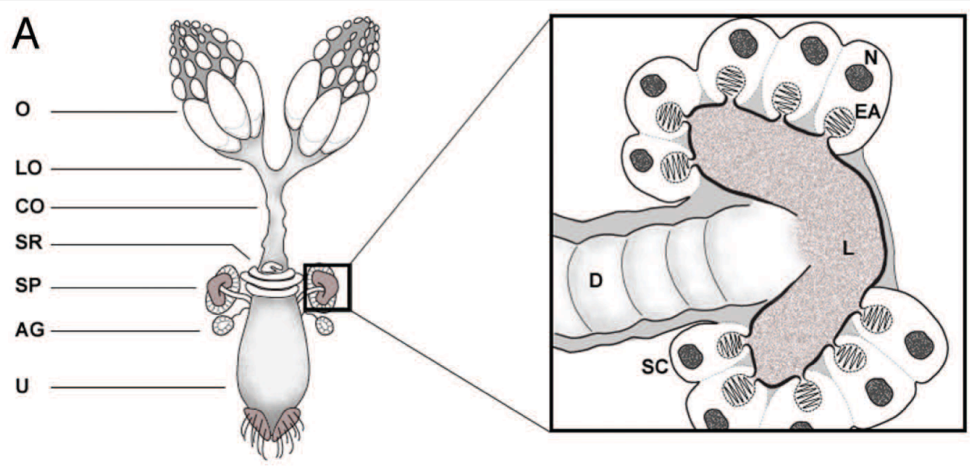

Figure 2. Overview of *D. melanogaster* female and male reproductive structures and glandular tissues. The female reproductive system is shown in ventral view, with anterior to the top. it contains a pair of ovaries (O), from which mature eggs pass to the lateral oviducts (LO), which join to form the common oviduct (CO). eggs are activated (Heifetz and Wolfner, 2001) in the common oviduct before passing to the uterus (U), where fertilization takes place. The entrance to the egg, or micropyle, is adjacent to the openings of the ducts to the spermathecae (SP) and seminal receptacle (SR). Aside from their role as SSOs, the spermathecae function as glandular structures. Each spermathecal duct (D), which is surrounded by a thin layer of muscle and epithelial tissue, leads from the anterior-dorsal uterus to the lumen (L) of a cuticular capsule where sperm are stored. Surrounding the capsule is a ring of polarized secretory cells (SC), with nuclei (N) distal to the capsule, that release the contents of the end apparatus (EA), a large membrane-rich secretory organelle, into the lumen (Allen and Spradling, 2008). Small accessory glands (AG) also connect through ducts to the anterior-dorsal uterus. Modified from Schnakenberg et al., 2012.

To identify these neurons, I make use of a well-established technique in *Drosophila*, the binary Gal4-UAS system (Figure 3) used for targeted gene expression (Brand and Perrimon, 1993). Flies have been genetically engineered to express Gal4, an 881 amino acid protein produced in yeast (*Saccharomyces cerevisiae*) that functions to regulate gene expression, and include an upstream activation sequence (UAS) within their genome.

The UAS is a cis-regulatory site (a sequence of DNA) in which the Gal4 protein binds to. The expression of Gal4 is under the control of a given *Drosophila* promoter, known as the driver, which allows for tissue specific expression. Alone, Gal4 expression has little influence, as the protein is not indigenous to *Drosophila*; and UAS in the *Drosophila* genome, by itself, also lays dormant. These two genetic constructs are generated in two different flies. By crossing males and females, each containing one part of the binary system, the offspring will contain both elements. Upon interaction with the UAS, Gal4 recruits genetic expression machinery and drives the expression of the gene of interest located downstream of the UAS (Figure 3; Johnston, 2002). Using this system I was able to drive the expression of temperature sensitive machinery is specific subpopulations of cells. At a permissive temperature (under 25°C), neurons expressing this machinery display normal activity. However, if the fly is placed above this threshold, neurons that express this machinery are forced to either fire or be silenced. Therefore, using the Gal4/UAS system it is possible to not only express this machinery in specific populations of neurons to identify if they support the specific post-mating behaviour, but also drive the expression of genes that allow for the identification of their projection pattern (Figure 3).

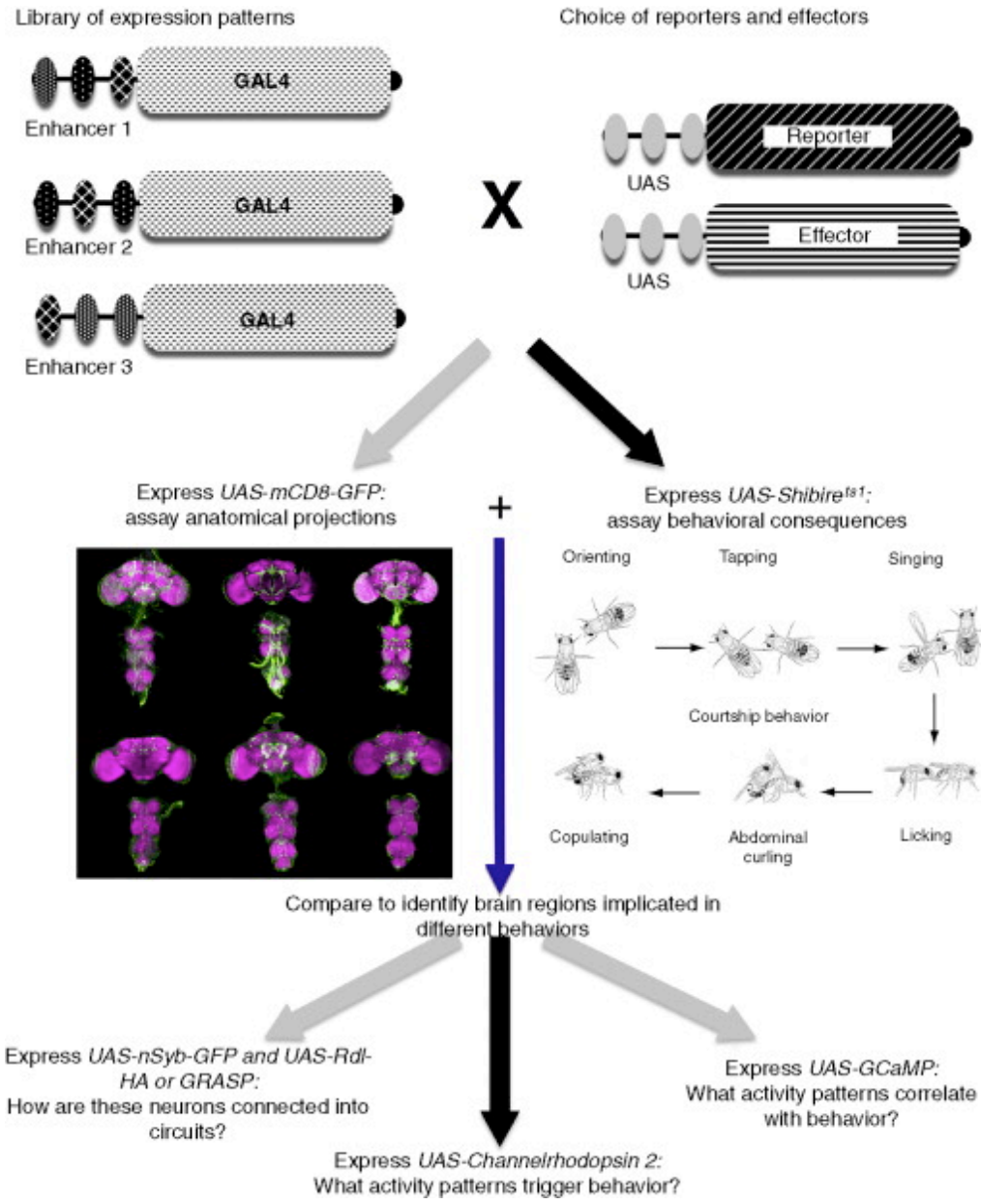

Figure 3. Screening approach to identify circuit components by targeted genetic lesions. A library of GAL4 lines is crossed to anatomical markers to determine the identity and potential connectivity of neurons. The same lines are crossed to neural activity blockers or activators and behavioral effects assayed. Lines that show similar behavior defects can be compared to look for shared neurons. Taken from Simpson, 2009.

THESIS OVERVIEW

In the case of polyandry, sexual conflict theory predicts that when females re-mate in an attempt to increase their personal fitness at the cost of their previous mate(s), males should develop adaptations to either reduce the chances of remating or succeed in sperm competition. In this thesis, I combine novel behavioural assays, gas chromatography, technically challenging dissections, thermogenetics, immunostaining, genetic cloning, and confocal microscopy to further our understanding of female post-mating behaviours in this species. I find evidence that females have indeed developed counter-adaptations in response to male tactics of chemical mate-guarding (**Chapter 3**) and paternity manipulation such as last male sperm precedence (**Chapter 4**). In order to provide evidence of a mechanism for cryptic control, I explored the neuronal substrate that supports two classes of sperm manipulation by females: sperm storage (**Chapter 5**) and sperm ejection (**Chapter 6**). Instead of focusing on only ultimate causes of behaviour or the proximate mechanisms that are involved with the central nervous system and behavioural output, I use these approaches to integrate these two perspectives. Together, I show that females can impose their influence over progeny production and have developed counter-adaptations to male-derived mechanisms intended on decreasing the cost of polyandry. I identify the potential neuronal substrate supporting sperm manipulation by females, providing evidence in support of nervous system-mediated mechanisms for cryptic female choice in this species. As the extensive review of the literature (**Chapter 2**) concerning female post-mating behaviour reveals many more questions about female PMR than hard facts, I encourage the integration of the two perspectives and attempt to show that they complement, as opposed to conflict.